

Ribosomal DNA ITS2 Sequences Differentiate Six Species in the *Anopheles crucians* Complex (Diptera: Culicidae)

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ABSTRACT *Anopheles crucians* Wiedemann (*sensu lato*) was investigated for the presence of cryptic species using rDNA ITS2 sequences. This complex of species presently contains the named species *An. crucians*, *An. bradleyi* King, and *An. georgianus* King. Adult female mosquitoes were collected at 28 sites in Alabama, Florida, Georgia, North Carolina, Mississippi, and Louisiana, resulting in 245 progeny broods. Species were identified using preliminary morphological characters, and the internal transcribed spacer two (ITS2) was amplified from all broods. The result was five distinct sizes of amplification product, and based on morphological characters, one of the size classes was suspected to consist of two species. All six putative species were then sequenced: five directly, and the sixth, because of extreme intragenomic (each individual with many variants) size variability, cloned. The ITS2 sequences were markedly distinct for all six species. Species designations and ITS2 sequence lengths (base pairs in parentheses) were A (461), B (1,000+), C (204), D (293), E (195), and *An. bradleyi* (208). Species B showed both large intraspecific and intragenomic sequence variability and is distinguished by having the longest ITS2 found so far in an *Anopheles*. Based on these data, we found that all species could be identified with polymerase chain reaction (PCR) using a mixture of four primers in a single reaction. Members of this complex were often found in sympatry, with the adults of five species collected at a single site in central Florida.

KEY WORDS *Anopheles*, *crucians*, internal transcribed spacer two, species complex, malaria

RI BOSOMAL DNA HAS BEEN used to answer systematics and phylogenetics questions in a wide variety of organisms (e.g., Miller et al. 1997 for Diptera). The functional regions that produce the ribosomes are highly conserved, while at the same time, there are transcribed and nontranscribed spacer regions that have high interspecific and low intraspecific variability, making them useful for study of relationships of closely related species and as a basis for polymerase chain reaction (PCR) identification of isomorphic species complexes. Ribosomal DNA exists as a tandem array of many copies per cell (Gerbi 1985). The relative homogeneity of this gene family is, like others, thought to be maintained by a variety of mechanisms collectively referred to as concerted evolution, where mutations rapidly spread to all members of the gene family even if there are arrays located on different

chromosomes (Arnheim 1983, Gerbi 1985, Tautz et al. 1988). In mosquitoes, each transcriptional unit is made up of an external transcribed spacer, an 18S subunit, an internal transcribed spacer one (ITS1), a 5.8S subunit, an internal transcribed spacer two (ITS2), and a 28S subunit. The transcribed spacers are thought to contain conserved structures, but not necessarily conserved sequence, important in forming the mature ribosomal amplicon (Gerbi 1985, Muller and Eckert 1989, Thweatt and Li 1990, Wesson et al. 1992, Paskewitz et al. 1993). In *Anopheles* mosquitoes, the ITS2 sequences of ≈ 103 species have either been published or are recorded in GenBank. ITS2 has been used often for both uncovering cryptic *Anopheles* species and as a source of species-specific PCR primers. Because of high interspecific variability and intraspecific homogeneity, the spacer sequences allow unambiguous species identification in a range of closely related mosquito species (Collins and Paskewitz 1996, Walton et al. 1999). Examples are numerous and include *An. quadrimaculatus* complex (Cornel et al. 1996); *An. gambiae* complex (Paskewitz et al. 1993, Scott et al. 1993); *An. dirus* complex; *An. bancroftii* group (Beebe et al. 2001); and *An. funestus* and related species (Hackett et al. 2000). Rarely, the ITS2 sequences from different cryptic species are too close to allow PCR-

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based differentiation; the only published example concerns *An. inundatus* Reinert (as C2) and *An. diluvialis* Reinert (as C1) of the *An. quadrimaculatus* complex (Cornel et al. 1996). For these reasons, we chose to investigate ITS2 sequences to distinguish members of the *An. crucians* complex.

Anopheles crucians Wiedemann is a common species in the southeastern and midwestern United States. The Crucians subgroup (Floore et al. 1976, Harbach 1994) of *Anopheles* subgenus *Anopheles* is presently made up of three named species: *An. crucians*, *An. bradleyi* King, and *An. georgianus* King. The three species are not currently separable in the adult female, although distinguishing pupal and larval characters have been published (Floore et al. 1976). *An. crucians sensu lato* is common in permanent and semipermanent ground pools in the United States (Massachusetts to New Mexico), in Central America (Mexico to Nicaragua), and on many Caribbean islands. *An. bradleyi* is usually found in brackish water along the Atlantic and Gulf coasts of the United States and also as far south as Nicaragua. *An. georgianus* is less well known and found in smaller water accumulations such as seepage areas, hoofprints, and potholes in the southeastern United States (Floore et al. 1976).

Anopheles crucians s.l. has been implicated as either a very good malaria vector or as a zoophilic nonvector (reviewed by Floore et al. 1976). In a recent occurrence of autochthonous *Plasmodium falciparum* infection, Strickman et al. (2000) hypothesized that *An. crucians* was not a likely vector in Colonial Beach, VA, because it was scarce in human landing collections. Numerous viruses have been isolated from *An. crucians* including EEE, SLE, Tensaw, VEE, Keystone, Trivittatus, LaCrosse, South River, West Nile, and Cache Valley (Floore et al. 1976 (review), Day and Stark 1996, Mitchell et al. 1996, Nayar et al. 2001, Wozniak et al. 2001, <http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm>). In addition, Parker (1993) concluded that *An. bradleyi* (an apparent presumptive identification based on its presence in a brackish water habitat) was the primary vector of *Dirofilaria immitis* (Leidy) at a site on coastal North Carolina.

Recently, it became evident that *An. crucians s.l.* was a species complex. Cockburn et al. (1993), using mitochondrial RFLPs and ribosomal DNA ITS2 size differences separated four species, designated "A," "B," "*An. bradleyi*," and "*An. bradleyi*?" Based on molecular data provided in the above paper, J.F.R. began raising progeny broods from various localities. Species hypotheses were then formulated based on preliminary molecular studies and morphological observations (to be published separately). We then explored rDNA ITS2 sequences for potential markers to confirm/refute these preliminary identifications.

Materials and Methods

Source of Specimens. Adult females were aspirated from resting sites or collected using CDC light traps, usually baited with dry ice. In addition, some adults

were obtained from individually reared larvae. There were 28 collection sites (Table 1; Appendix) in six states: Alabama, Florida, Georgia, Louisiana, North Carolina, and Mississippi. The methods of Reinert et al. (1997) were followed for collection and progeny rearing. These collections resulted in 245 progeny broods and an additional six adults reared from larvae. An adult representative from each progeny brood and the six individually reared larvae were evaluated. Progeny broods were preserved both for morphological study (paper pinpointed adults with associated larval and pupal exuviae in 80% ethyl alcohol for slide mounting) and for molecular study (frozen at -80°C). The frozen specimens used in this study were later placed in 100% ethyl alcohol before DNA extraction.

Mosquito Identification. Adult females were classified as belonging to the Crucians subgroup using the characters summarized in Floore et al. (1976). Most specimens were tentatively separated into one of the following six morphospecies: *bradleyi*, sp. A, sp. B, sp. C, sp. D, or sp. E. Morphological separation was based on preliminary characters to be finalized separately. It is not possible at this time to determine which of the lettered species refer to the other two named species in this complex, *An. crucians* and *An. georgianus*. Voucher specimens from the morphological study will be deposited in the National Museum of Natural History, Smithsonian Institution, Washington DC; the Florida State Collection of Arthropods, Division of Plant Industry, Florida State Department of Agriculture, Gainesville, FL; and The Natural History Museum, London, United Kingdom.

DNA Isolation. DNA was isolated from individual adult mosquitoes by phenol-chloroform extraction as described in Wilkerson et al. (1993).

PCR Amplification. The ITS2 region was amplified using PCR primers based on conserved sequences in the 5.8S and 28S ribosomal subunits of *An. quadrimaculatus* complex species (Cornel et al. 1996): ITS2 F 5' TGTGAAGTGCAGGACACATGAA 3' and ITS2R 5' ATGCTTAAATTTAGGGGGTACTC 3'. The boundaries of the ITS2 were determined using the predicted base pairings between the ends of the 5.8S and 28S ribosomal subunits that are figured by Cornel et al. (1996) for *An. quadrimaculatus* complex species A (*An. quadrimaculatus*). Reactions were carried out in a total volume of 50 μl using the PCR buffer (GeneAmp 10 \times Buffer II) supplied with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). Final reaction concentrations were 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.0 mM MgCl_2 ; 0.25 mM each of dATP, dCTP, dGTP, and dTTP; 15 pmol of each primer; 0.2–4.0 ng template DNA (1/100 of DNA from entire adult mosquito)/reaction; and 1.25 U of AmpliTaq/reaction. A Perkin Elmer Applied Biosystems 9700 thermocycler was used for PCR with the following parameters: initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR product was separated on a

Table 1. Collection localities and species identifications.

Collection locality	Species					
	Total number of families (number sequenced)					bradleyi
	A	B	C	D	E	
1. Alabama, Mobile Co., Mobile 30° 22' 16" N, 88° 09' 50" W (AL94.0)						8 (1)
2. Alabama, Lauderdale Co. 34° 48' 50" N, 87° 50' 52" W (AL99.1)					7	
3. Florida, Alachua Co., Paynes Prairie 29° 36' 24" N, 82° 18' 14" W (FL95.36)		1 (1)				
4. Florida, Levy Co., nr Chiefland 29° 29' 07" N, 82° 58' 37" W (FL95.49)					1 (1)	
5. Florida, Alachua Co., Gainesville 29° 38' 37" N, 82° 21' 30" W (FL95.70)		4	1			
6. Florida, Alachua Co., nr Micanopy 29° 31' 34" N, 82° 16' 30" W (FL95.69, FL97.34, FL97.60)	32 (1)	1	12 (2)	3 (2)	1	
7. Florida, Jefferson Co., nr Ashville 30° 35' 39" N, 83° 43' 06" W (FL95.117)	2		6		1	
8. Florida, Baker Co., nr Baxter 30° 31' 02" N, 82° 13' 50" W (FL97.11)	7					
9. Florida, Nassau Co., nr Jacksonville 30° 34' 31" N, 81° 38' 45" W (FL97.13)						3
10. Florida, Alachua Co., Gainesville 29° 43' 15" N, 82° 23' 54" W (FL98.17, FL99.1)	17			4	7	
11. Florida, Jefferson Co. 30° 26' 42" N, 83° 43' 27" W (FL98.27)	1					
12. Florida, Levy Co. 29° 13' 08" N, 83° 00' 58" W (FL99.32)						5
13. Florida, Alachua Co., Gainesville 29° 42' 00" N, 82° 18' 20" W (FL98.8)	1					
14. Florida, Brevard Co., Merritt Island 28° 26' 00" N, 80° 41' 44" W (approx.) (SYK1, SYK2)						2
15. Georgia, Brooks Co., nr Quitman 30° 48' 01" N, 83° 32' 19" W (GA95.25)	4 (1)		1 (1)		4 (1)	
16. Georgia, Camden Co., Woodbine 30° 58' 31" N, 81° 43' 34" W (GA97.4)		2				
17. Georgia, Chariton Co., Folkston 30° 47' 25" N, 81° 58' 58" W (GA97.5)				1		
18. Georgia, Brooks Co. 30° 40' 13" N, 83° 24' 00" W (GA98.1, GA98.10)	2 (2)			2 (2)		
19. Georgia, Lowndes Co. 30° 55' 30" N, 83° 02' 08" W (GA98.16)	1			2 (2)		
20. Georgia, Tifton Co. 31° 28' 23" N, 83° 39' 49" W (GA98.29)	1					
21. Louisiana, Orleans Parish, New Orleans 30° 03' 16" N, 89° 52' 21" W (LA95.6)		10	1		1	1 (1)
22. Louisiana, Orleans Parish, New Orleans 30° 06' 55" N, 89° 51' 57" W (LA95.7)		1				
23. Louisiana, Orleans Parish, New Orleans 30° 04' 43" N, 89° 55' 34" W (LA95.8)		9			4	
24. Louisiana, Calcasieu Parish, Moss Bluff 30° 18' 01" N, 93° 11' 15" W (approx.) (LA98.1, LA98.2)	1		1		3	
25. Louisiana, Orleans Parish, New Orleans 30° 04' 33" N, 89° 55' 12" W (LA95.9)		7			5	
26. Louisiana, Calcasieu Parish, Moss Bluff 30° 18' 00" N, 93° 10' 56" W (approx.) (LA98.3, LA98.4)		2			1	
27. Mississippi, Tishomingo Co., nr Tishomingo 38° 38' 26" N, 88° 10' 08" W (MS99.1)	2				6	
28. North Carolina, Rowan Co., Salisbury 35° 41' 25" N, 80° 28' 21" W (NC96.1)	4 (1)				39 (1)	
Total	75	37	22	12	80	19
Sequenced	5	1	3	6	3	2
						245

See Appendix for detail.

2.5% agarose gel and visualized with ethidium bromide stain. Fragment sizes were estimated by comparison to molecular weight standards provided by lambda DNA digested by *Hind*III and phiX174 DNA digested with *Hae*III (Sigma, St. Louis, MO). Based on sequencing results (Fig. 1), two additional internal primers were designed to distinguish the three species with similar

size ITS2 amplification products (*An. bradleyi*, C, and E): *An. bradleyi* reverse 5'TCTAGCTGTC-CATCTCGTCG3' and species C reverse 5'AAGC-CCCTTCTTACTCCATC3'. The PCR reagents and conditions were the same when all four primers were included in a reaction, except 10 pmol of each of the additional two primers were included.

Fig. 1. *Anopheles crucians* complex rDNA ITS2 sequence. Asterisks mark the start and end of the ITS2 based on presumed complementarity of the 3' end of the 5.8S and 5' end of the 28S subunits as described in text. Arrows and underlined sequence indicate the ITS2 forward and reverse primers and the species C and *bradleyi* reverse primers. Species B positions 373–672 are in the boxed area.

and the sequences were generated with an ABI 377 automated sequencer. The sequences were analyzed, and questionable base calls were resolved using Sequencher 3.0. The sequence was compared and visually aligned using Se-Al version 2.0a9 (Sequence Alignment Editor; A. Rambaut, Univ. of Oxford) or

MacClade (Maddison and Maddison 2000). Sequence statistics were obtained using PAUP version 4.0b4 (Swofford 1998).

Cloning of Species B. Because the ITS2 amplification product for species B consisted of numerous different size products, it was necessary to clone individual ITS2s to obtain unambiguous sequence. An individual from each of three progeny broods from widely separated localities was chosen for cloning (FL95.75, GA97.7, LA95.25). The PCR products were amplified by primers ITS2 F and ITS2R and cleaned using QIAquick PCR purification kit (Quiagen, Valencia, CA). Approximately 200 ng of each purified PCR product was ligated into pCR-TOPO plasmid (Invitrogen, Carlsbad, CA). Two microliters of the ligation reaction mixture was transformed into competent One Shot cells (TOPO TA Cloning Kit; Invitrogen). Transformed cultures were plated overnight on LB plates containing X-Gal, isopropylthio-beta-D-galactoside (IPTG) and 50 µg/ml ampicillin. Eighteen clones were picked from FL95.75, and 10 each from GA97.7 and LA95.25. Successful insertions were confirmed by PCR. Plasmids were extracted by the miniprep method (Sambrook et al. 1989). Each cloned insert was sequenced in both directions with M13 (Invitrogen) forward and reverse primers. Sequencing and alignment were conducted as above.

Results

PCR amplification of the rDNA ITS2 resulted in five distinct size classes of amplification product (Fig. 2). These usually corresponded to preliminary morphological identifications and were given the species designations A, B, D, E, and *An. bradleyi*/C. The *An. bradleyi*/C size class suggested a barely discernible size difference between these two putative species. Further separation was attempted using MetaPhor agarose (FMC BioProducts) with inconclusive results (data not shown). Note that to confidently distinguish the *An. bradleyi*/C size class from E, it is necessary to run them next to each other and to allow migration of the fragments as far as possible on the gel.

Sequences obtained from three to six individuals of each putative species (Fig. 1; Table 2) showed distinct and consistent base differences among all species, including *An. bradleyi* and C, whose overall length difference was only 4 bp. To illustrate the consistency of ITS2 lengths, we present results from three individuals from widely separated collection sites in Fig. 2. Direct sequencing was possible from species A, C, D, E, and *An. bradleyi* (GenBank AY245553, AY386965, AY386964, AY386966, and AY386967, respectively). Sequences from the directly sequenced species showed no intraspecific differences in the sample tested, nor were there any indications of intragenomic variation (a single individual with many different ITS2 lengths) that would have resulted in many doubtful base calls and superimposed chromatogram peaks. Species B, with the largest ITS2, appeared on the gel as a blur with several more or less distinct imbedded

bands. The 38 cloned amplification fragments from species B ranged from 1,006 to 1,218 bp. (Fig. 1; Table 2; GenBank AY386963). The large sequence difference between species B and the other five species, and the species B intragenomic variability, was the result of a complex array of repeats (7–82 bp) in different combinations. Although some of the size and repeat combinations are more common than others, about one-half of the clones also showed differences caused by a small number of mutations or indels (unpublished data).

The extreme ITS2 size differences between species A, D, and B, compared with *An. bradleyi*, C, and E (Fig. 2), along with sequence differences (Fig. 1), allowed us to use four primers in a single reaction for identification of all six species (Fig. 3; Table 3). Species D and E can be recognized by single bands from amplification by ITS2 F and R primers alone. Species B is recognized by a blurred band described above, also from amplification by the ITS2 F and R primers. Species A is recognized by the band produced by amplification of ITS2 F and R primers and by a second band resulting from the ITS2 F primer and the internal *An. bradleyi* R primer, resulting from amplification of sequence homologous with *An. bradleyi*, but beginning at position of 514 of species A). In some reactions (data not shown), probably because of slight variation in reaction conditions, there was also a third band produced, even though the bases did not all match (incomplete complementarity) with the species C reverse primer. *An. bradleyi* is recognized by the amplification product from the ITS2 F and R primers, and another slightly smaller product, which is faint but consistent, from the ITS2 F primer and internal *An. bradleyi* R primer. Finally, species C can be recognized by the band resulting from the ITS2 F and R primers, plus another faint but consistent band produced by ITS2 F and the internal species C-specific primers. The species C band is the same size as the one sometimes amplified in species A. Therefore, species identifications in the case of species A, *An. bradleyi*, and species C depend on the presence of a combination of bands, not single species-specific markers. Consistency of results is illustrated in Fig. 3 using pairs of individuals of each species from widely separated collection sites.

Using the above primers, we were able to identify to species all 245 families and six individual rearings, as shown in the examples on Fig. 3. In all, there were 75 A, 37 B, 22 C, 12 D, 80 E, and 19 *An. bradleyi* (Table 1; Appendix). Fourteen of the 28 collection sites had two or more species occurring together, demonstrating sympatry of all combinations of species. One collection site, Micanopy, FL, had all species except *An. bradleyi*. *An. bradleyi* was not found at this site probably because it is a known coastal species with immatures breeding in brackish water and would be unlikely to be found inland. However, it was sympatric with species B, C, and E at site 21, New Orleans, LA, from a light trap collection near both brackish and fresh water habitats (see Table 1 for details on co-occurrence of species).

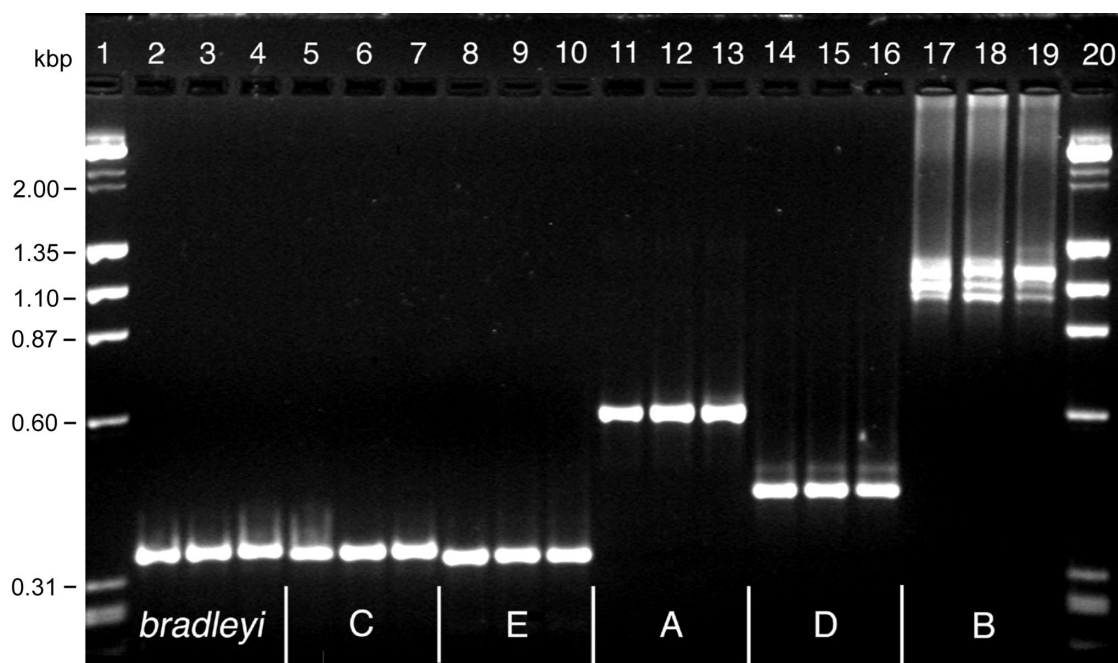


Fig. 2. PCR product from amplification of the rDNA ITS2 of *An. crucians* complex species using conserved primers in the 5.8S (forward) and 28S (reverse) subunits. See text and Table 2 for explanation of species names and fragment sizes. Lanes 1 and 20 are DNA size ladder; labeled bands are in kilobase pairs (kbp).

GC content of the ITS2 reported here (50–56%) is consistent with spacer base composition in other *Anopheles* with a few exceptions: two *Anopheles dirus* complex species (69%) (Xu and Qu, 1997) and in the *An. punctulatus* group (Beebe et al. 1999) (70%).

Discussion

Here we demonstrate, using the rDNA ITS2 sequence, that the *Anopheles* Crucians Complex is composed of six species. Unambiguous sequence differences among them allowed us to use only four primers in a single reaction to identify all six. Morphologically, only *An. bradleyi* can be separated with certainty at this time.

The phenomenon of lack of correspondence between morphological similarity and similarity in the bionomics, vector potential, or insecticide resistance is well documented in *Anopheles* mosquitoes. An excellent example is the African *An. gambiae* complex (White 1974, Coluzzi et al. 1979). Only by knowing the genetic identity of the organism under investigation can this particular part of an experimental design be accounted for. For example, the results of studies on photoperiod and longevity (Lanciani 1993), trap effectiveness (Kline et al. 1991), larval distribution (Rejmankova et al. 1993), or general ecology (Hu et al. 1993) could all have been affected by the presence of more than one species being included under *An. crucians*. Furthermore, subsequent studies or control efforts based on the results from experiments on a mixture of cryptic species could be faulty because of

erroneous or skewed results. The single PCR reaction method using the four primers described here will allow for unambiguous identification of wild-caught Crucians Complex species in future studies.

It is apparently possible to identify larval and pupal *An. bradleyi* using the morphological characters found in Floore et al. (1976), but it is not known at this time which of the other taxa might be *An. crucians* s.s., *An. georgianus*, or an as yet unnamed species.

Published sequences (e.g., Paskewitz et al. 1993, Cornel et al. 1996, Fritz 1998, Manguin et al. 1999, Marinucci et al. 1999, Linton et al. 2002) and unpublished GenBank submissions show that most *Anopheles* ITS2s are ≈300–600 bp. Slightly larger are *An. dirus*, *An. nemophilus*, *An. dirus* B, and *An. dirus* C, which are in the 733–800 range (Walton et al. 1999). Species B has the longest ITS2 (over 1,000 bp) reported so far for any *Anopheles*, whereas all other Crucians Complex species range from 195 to 461 bp.

Table 2. *An. crucians* complex rDNA ITS2 sequence statistics

Species	n	ITS2 (bp)	GC (%)	Amplified fragment bp ^a
A	5	461	54.32	595
B clone 1–6	—	1021	51.36	1,155
B clone 1–10	—	872	50.17	1,006
C	3	204	53.65	338
D	6	293	54.08	427
E	3	195	56.12	329
<i>bradleyi</i>	3	208	54.32	342

^a ITS2 plus 91 bp at end of 5.8S and 43 bp from beginning of 28S.

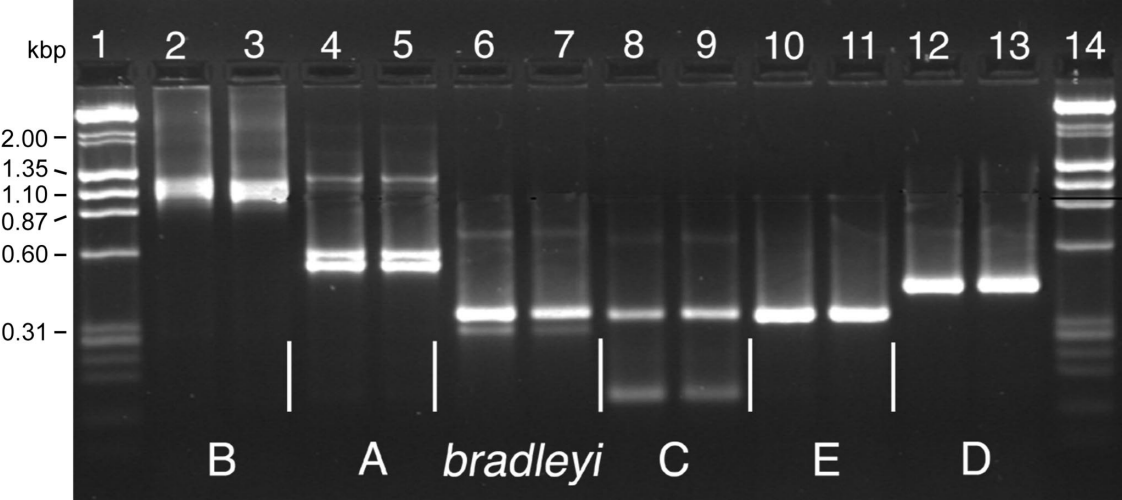


Fig. 3. PCR amplification products for identification of the six species in the *An. crucians* complex generated by multiplexing four primers. The position and sequence of the primers, ITS2 forward and reverse primers and the species C and *bradleyi* reverse primers, can be found in Fig. 1 and Tables 2 and 3. Lanes 1 and 14 are DNA size ladder; labeled bands are in kilobase pairs (kbp).

Species B ITS2 is unusual in another way because it exhibits abundant intragenomic variation. In-tragenomic variation of ITS2 in *Anopheles* is documented and may be common, e.g., Onyabe and Conn (1999) for *An. (Nys.) nuneztovari* Gabaldon. In their review, they note its occurrence in other *Anopheles* and that there is a large amount of variation in genera *Aedes* and *Culex* (Black et al. 1989, Wesson et al. 1992, Miller et al. 1996). However, these instances of variation are caused by a low number of indels or the presence or absence of a low number of repeats. While a possible confounding factor in phylogenetic analyses, it does not suggest a breakdown of concerted evolution. Species B ITS2 intragenomic length variability does, however, suggest that this is the case.

The Crucians Complex ITS2 size differences reported by Cockburn et al. (1993) can with fair certainty be related to the species reported here: our

species B, A, D, and *An. bradleyi* correspond to their species “A,” “B,” “*An. bradleyi*?,” and “*An. bradleyi* s.s.,” respectively. Although no sequences were obtained by them and amplification products were estimated on agarose gels, the approximate amplification sizes (including flanking regions of the 5.8S and 28S) were consistent with our results.

In conclusion, we believe that our data support hypotheses of reproductive isolation and that these taxa represent valid species for the following reasons: (1) there are at least two species sympatric at 14 of 28 collections sites, with five sympatric at 1 site; (2) there is no suggestion of hybridization, i.e., multiple ITS2 sequences in the same individual belonging to different clades (as reported for *Culex pipiens* ITS1 by Miller et al. 1996); (3) preliminary morphological identifications (data not shown) were almost always in agreement with the molecular data, i.e., correlated characters from a morphological data set; and (4) ITS2 sequence variability among the Crucians Complex species is larger than that found in some other *Anopheles* cryptic species groups, which when considered with the above, is an indication that good biological species are represented.

Table 3. *An. crucians* complex ITS2 fragment sizes (bp) generated by multiplexing conserved ITS2 forward and reverse primers, and species C and *bradleyi* reverse primers

Species	Fragment 1 ^a	Fragment 2	Fragment 3
B	1,000+	—	—
A	595	533 ^b	147 ^c
<i>bradleyi</i>	342	295 ^d	—
C	338	147 ^c	—
D	427	—	—
E	329	—	—

^a Amplification product of ITS2 forward and reverse primers.
^b Amplification product of ITS2 forward and *bradleyi* reverse primers.
^c Sometimes amplified by ITS2 forward and species C reverse primers (not figured).
^d Amplification product of ITS2 forward and *bradleyi* reverse primers.
^e Amplification product of ITS2 forward and species C reverse primers.

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Appendix

Anopheles crucians complex, detailed collection information. See Table 1 for state, county, municipality, coordinates and collection number of each collection site. Each site has one or more unique collection number(s) consisting of a state indicator and number, e.g., AL94.0. Each individual progeny brood (IPB) from a collection has a designator consisting of the state (two letters) and a unique number (last two digits of year collected followed by a period and the collection), e.g., AL94.1, this is followed by a species identification in parentheses. Collector's were Bruce A. Harrison (BAH); Daniel L. Kline (DLK); John F. Reinert (JFR); Kenneth J. Tennessen (KJT); Karl R. Kangas (KRK); Osborne R. Willis (ORW); Parker B. Whitt (PBW); Paul E. Kaiser (PEK); Scott F. Willis (SFW); Scott G. Straub (SGS); and Tokuo Fukuda (TF).

Locality 1. 11 November 1994, PEK coll., West Fowl River near Road 188, CDC light trap. Coll. No. AL94.0, IPBs: AL94.1 (*bradleyi*); AL94.2 (*bradleyi*); AL94.3 (*bradleyi*); AL94.4 (*bradleyi*); AL94.6 (*bradleyi*); AL94.7 (*bradleyi*); AL94.8 (*bradleyi*); and AL94.9 (*bradleyi*).

Locality 2. 10 June 1999, KJT coll., Sinking Creek Swamp, ≈7 miles west of Florence, CDC light trap + dry ice, Coll. No. AL99.1, IPBs: AL99.2 (E); AL99.3 (E); AL99.4 (E); AL99.7 (E); AL99.8 (E); and AL99.9 (E).

Locality 3. 24 August 1995, JFR and PEK colls., Paynes Prairie State Preserve, north rim at park HQ, females collected resting in horse barn, Coll. No. FL95.36, IPB: FL95.38 (B).

Locality 4. 31 August 1995, JFR and PEK colls., Manatee Springs State Park, Magnolia Camp-SW, female collected resting in rot cavity of tree, Coll. No. FL95.49, IPB: FL95.50 (E).

Locality 5. 31 October 1995, JFR and PEK colls., University of Florida campus, wooded area north side of Lake Alice, CDC light trap + dry ice, Coll. No. FL95.70, IPBs: FL95.72 (B); FL95.73 (B); FL95.74 (C); FL95.75 (B); and FL95.76 (B).

Locality 6. 31 October 1995, JFR and PEK colls., 1 mile NE of U.S. 441 on CR 234, CDC light trap + dry ice, Coll. No. FL 95.69, IPBs: FL95.77 (C); FL95.79 (C); FL95.82 (A); FL95.83 (C); FL95.87 (A); FL95.88 (A); FL95.89 (A); FL95.90 (A); FL95.91 (A); FL95.92 (A); FL95.94 (C); FL95.95 (A); FL95.98 (A); FL95.99 (C); FL95.101 (D); FL95.103 (C); FL95.104 (C); FL95.105 (C); FL95.108 (A); FL95.109 (A); FL95.110 (A); FL95.111 (A); FL95.112 (C); FL95.113 (A); FL95.114 (A); and FL95.133 (D); same except 20 August 1997, JFR coll., two CDC light traps + dry ice, Coll. No. FL97.34, IPBs: FL97.35 (E); FL97.36 (A); FL97.37 (A); FL97.38 (B); FL97.39 (A); FL97.40 (A); FL97.41 (A); FL97.42 (A); FL97.43 (C); FL97.46 (A); FL97.47 (A); FL97.48 (A); FL97.49 (A); FL97.50 (A); FL97.51 (A); FL97.52 (A); FL97.53 (A); FL97.54 (C); FL97.55 (A); FL97.56 (A); and FL97.57 (C); same except 4 November 1997, JFR coll., larvae collected from water in grassy roadside barrow ditch, Coll. No. FL97.60, individually reared specimens: FL97.60 (A).

Locality 7. 29 November 1995, JFR and PEK colls., west of Ashville on CR 146 near bridge over Aucila River, two CDC light traps + dry ice, Coll. No. FL95.117, IPBs: FL95.119 (C); FL95.120 (C); FL95.123 (C); FL95.124 (C); FL95.125 (C); FL95.126 (E); FL95.127 (A); FL95.128 (A); and FL95.135 (C).

Locality 8. 20 May 1997, JFR and KRK colls., Hwy two bridge over St. Marys River, adults resting under bridge, Coll. No. FL97.11, IPBs: FL97.15 (A); FL97.17 (A); FL97.20 (A); FL97.21 (A); FL97.22 (A); FL97.24 (A); and FL97.25 (A).

Locality 9. 20 May 1997, JFR and KRK colls., I-95 bridge over Nassau River, adults resting under north end of bridge, Coll. No. FL97.13, IPBs: FL97.16 (*bradleyi*); FL97.18 (*bradleyi*); and FL97.23 (*bradleyi*).

Locality 10. 8 March 1998, DLK coll., 5027 NW 75th Lane, counter flow trap + CO₂ + no light, Coll. No. FL98.17, IPBs: FL98.18 (A); FL98.19 (A); and FL98.20 (A); same except 19 March, 25 March 1999, Coll. No. FL99.1, IPBs: FL99.2 (E); FL99.3 (E); FL99.4 (A); FL99.5 (E); FL99.6 (E); FL99.7 (A); FL99.8 (E); FL99.9 (A); FL99.10 (A); FL99.11 (D); FL99.12 (E); FL99.13 (D); FL99.14 (D); FL99.15 (A); FL99.16 (A); FL99.17 (A); FL99.18 (A); FL99.19 (E); FL99.20 (A); FL99.21 (A); FL99.23 (D); FL99.24 (A); FL99.25 (A); FL99.26 (A); and FL99.27 (A).

Locality 11. 14 April 1998, JFR and ORW colls., I-10 bridge over Aucilla River, adults resting under bridge, Coll. No. FL98.27, IPB: FL98.29 (A).

Locality 12. 3 September 1999, DLK coll., Lower Suwanee Wildlife Refuge, South Cabin Road, ≈23 miles N of Cedar Key, downdraft trap + CO₂ + no light, Coll. No. FL99.32, IPBs: FL99.33 (*bradleyi*); FL99.35 (*bradleyi*); FL99.36 (*bradleyi*); FL99.37 (*bradleyi*); and FL99.39 (*bradleyi*).

Locality 13. 22 January 1998, ORW and TF colls., on NE 15th Street south of NE53rd Avenue, water in roadside barrow ditch, Coll. No. FL98.8, individually reared specimens: FL98.8 (A).

Locality 14. 7 May 1993, PEK, Brevard Co., Merritt Island, Sykes Creek, CDC light trap+dry ice, Coll. SYK, IPBs: SYK1 (*bradleyi*) and SYK2 (*bradleyi*).

Locality 15. 27 March 1995, PEK and SGS colls., north of Quitman on Hwy 76 across Okapilco River at Hatch Ranch, CDC light trap + dry ice, Coll. No. GA95.25, IPBs: GA95.3 (A); GA95.5 (A); GA95.9 (A); GA95.10 (E); GA95.11 (E); GA95.14 (A); GA95.15 (C); GA95.16 (E); and GA95.19 (E).

Locality 16. 20 May 1997, JFR and KRK colls., US17 bridge over Satilla River, adults resting under bridge, Coll. No. GA97.4, IPBs: GA97.7 (B) and GA97.8 (B).

Locality 17. 20 May 1997, JFR and KRK colls., US301 bridge at mile marker 1.5, adults resting under bridge, Coll. No. GA97.5, IPB: GA97.9 (D).

Locality 18. 14 April 1998, JFR and ORW colls., north side of Nankin-Clyattville Road, 0.4 mile west of third bridge over Withlacoochee River, Coll. No. GA98.1, individually reared specimens: GA98.1–69 (A) and GA98.1–143 (D); same except 16 April 1998, Coll. No. GA98.10, individually reared specimens: GA98.10–104 (D) and GA98.10–159 (A).

Locality 19. 16 April 1998, JFR and ORW colls., ≈0.1 mile east on US84, bridge over Alapaha River, adults resting under bridge, Coll. No. GA98.16, IPBs: GA98.19 (A); GA98.20 (D); and GA98.21 (D).

Locality 20. 28 April 1998, JFR and ORW colls., US82 bridge over Ty Ty Creek, ≈0.1 mile east of Worth Co. line, adults resting under bridge, Coll. No. GA98.29, IPB: GA98.29 (A).

Locality 21. 27 June 1995, PEK coll., Bayou Sauvage HQ on US90, behind HQ building, CDC light trap + dry ice, Coll. No. LA95.6, IPBs: LA95.10 (B); LA95.12 (B); LA95.13 (B); LA95.14 (B); LA95.40 (E); LA95.43 (*bradleyi*); LA95.45 (B); LA95.46 (B); LA95.48 (C); LA95.52 (B); LA95.59 (B); LA95.60 (B); and LA95.66 (B).

Locality 22. 27 June 1995, PEK coll., from Bayou Sauvage HQ on US90 turn north on US11 ≈3 miles, CDC light trap + dry ice, Coll. No. LA95.7, IPB: LA95.16 (B).

Locality 23. 27 June 1995, PEK coll., I-10 first exit east of I-510, off NW exit ramp, one CDC light trap + dry ice, Coll. No. LA95.8, IPBs: LA95.17 (B); LA95.19 (B); LA95.21 (B); LA95.22 (B); LA95.23 (B); LA95.42 (E); LA95.44 (E); LA95.50 (B); LA95.57 (B); LA95.61 (B); LA95.64 (B); LA95.65 (E); and LA95.67 (E).

Locality 24. 11 June 1998, SFW, small town ≈10 miles north of I-10 on US171, two CDC light traps + dry ice, Coll. Nos. LA98.1 and LA98.2, IPBs: LA98.7 (E); LA98.8 (A); LA98.9 (C); LA98.11 (E); LA98.12 (E).

Locality 25. 27 June 1995, PEK coll., I-10 first exit east of I-510, off SE exit ramp, Coll. No. LA95.9, IPBs: LA95.24 (E); LA95.25 (B); LA95.26 (E); LA95.27 (E); LA95.28 (B); LA95.29 (B); LA95.39 (B); LA95.41 (E); LA95.47 (B); LA95.49 (B); LA95.56 (B); and LA95.68 (E).

Locality 26. 11 June 1998, SFW, small town ≈10 miles north of I-10 on US171, two CDC light traps + dry ice, Coll. Nos. LA98.3 and LA98.4, IPBs: LA98.5 (B); LA98.6 (E); and LA98.10 (B).

Locality 27. 16 June 1999, KJT, Tishomingo Co., Gist Swamp, 3 miles east of Tishomingo, CDC light trap + dry ice, Coll. No. MS99.1, IPBs: MS99.2 (A); MS99.3 (E); MS99.4 (A); MS99.5 (E); MS99.6 (E); MS99.7 (E); MS99.9 (E); and MS99.10 (E).

Locality 28. 19 June 1996, BAH and PBW, Catawba College Ecological Preserve, CDC light trap + dry ice, Coll. No. NC96.1, IPBs: NC96.2 (E); NC96.3 (E); NC96.4 (E); NC96.5 (E); NC96.6 (E); NC96.7 (E); NC96.9 (E); NC96.10 (E); NC96.11 (E); NC96.12 (E); NC96.13 (E); NC96.14 (E); NC96.15 (A); NC96.16 (A); NC96.17 (E); NC96.18 (E); NC96.19 (A); NC96.20 (A); NC96.21 (E); NC96.22 (E); NC96.23 (E); NC96.24 (E); NC96.26 (E); NC96.27 (E); NC96.28 (E); NC96.29 (E); NC96.30 (E); NC96.31 (E); NC96.33 (E); NC96.34 (E); NC96.35 (E); NC96.36 (E); NC96.37 (E); NC96.38 (E); NC96.39 (E); NC96.40 (E); NC96.41 (E); NC96.42 (E); NC96.44 (E); NC96.45 (E); NC96.46 (E); NC96.47 (E); and NC96.48 (E).